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WITNESS my hand this
Fourteenth day of October 2004

J. Billingsley

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AUSTRALIA
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PROVISIONAL SPECIFICATION

Invention Title: THERAPEUTIC METHOD

Applicant: MONASH UNIVERISTY

The invention is described in the following statement:

THERAPEUTIC METHOD

Technical Field

The present invention relates to the therapeutic use of activin and/or follistatin to modify inflammatory diseases and improve patient responses.

Background Art

Inflammation is a complex multifaceted process in response to disease or injury which is regulated by the release of a series of cytokines (1). These cytokines are classified in general terms as pro- or anti-inflammatory cytokines and the critical balance between release and activity of cytokines with opposing actions regulates the inflammatory response to prevent it from becoming overt or understated.

If the inflammatory response continues unchecked and is overt then the host may suffer associated tissue damage and in severe cases this may present as septic shock and multi-organ failure can occur (2). Conversely, a poor or understated inflammatory response may mean uncontrolled infection resulting in chronic illness and host damage. Regulation of the inflammatory response is important at both the systemic level and the local level.

Activin is a multifunctional dimeric protein consisting of two activin β_A subunits and is a member of the transforming growth factor-β (TGF-β) superfamily (3). First isolated as a regulator of FSH release activin, or more specifically activin A, has been implicated in a number of inflammatory disorders and is now recognized as a true cytokine (3). To control its function, Activin A has a biological regulator follistatin, a monomeric protein that binds activin with high affinity and is believed to fate activin for lysosomal degradation (4).

Elevated concentrations of activin and aberrant expression has been associated with inflammatory states such as rheumatoid arthritis, wound healing and inflammatory bowel disease (5-7). Furthermore, levels of circulating activin A are elevated in patients with viral hepatitis, septicemia and meningitis (8-10). In *in vitro* studies, Activin A has the ability to antagonize a number of pro-inflammatory cytokines including IL-6 and IL-1β (11-14). Due to its apparent involvement in a

number of inflammatory states it has been proposed that activin may be involved in modulating inflammatory responses (15).

Although activin A is associated with an increasing number of inflammatory diseases, only limited data exist investigating its possible role in the classical inflammatory response. To date, this work has been limited to *in vitro* analysis. Analysis of pregnancy membrane explants demonstrated that low concentrations of activin A stimulated IL-6 production while high doses of activin A were inhibitory (16). Other studies demonstrated that activin A inhibited endogenous IL-6 production in thymocytes but in the presence of an excess of IL-6 stimulated thymocyte growth (12). These *in vitro* data indicate that both the amount of activin A present in the system, and the context in which it is released, determines if it acts in a pro- or anti-inflammatory manner. Our group has previously described the release of activin A in an ovine model of acute inflammatory challenge employing lipopolysaccharide (LPS) as the inflammatory stimulant (17). It was demonstrated that activin A was released rapidly after LPS challenge and concurrently with tumour necrosis factor-α (TNFα), peaking within an hour. We have recently confirmed this release profile in a similar mouse model (these data are currently unpublished pending the filing of this application: either don't use a citation for this or we can take out somehow). While the mechanisms of activin A release in response to inflammatory challenge is now established, the role of activin A release remains unclear.

The above discussion of background art is included to explain the context of the present invention. It is not to be taken as an admission that any of the documents or other material referred to was published, known or part of the common general knowledge in Australia at the priority date of any one of the claims of this specification.

Description of the Invention

Throughout the description and claims of this specification, the word "comprise" and variations of that word, such as "comprising" and "comprises" are not intended to exclude other additives, steps or integers.

This study investigated the function of activin A in an acute inflammatory response. To investigate this we employed follistatin, the biological regulator of activin, specifically the 288 amino acid isoform which demonstrates a high affinity for heparan sulphate proteoglycans on cell surfaces and is proposed to fate activin, once bound, to clearance from the system via a lysosomal degradation pathway. We report here that administration of follistatin prior to an injection of LPS, whilst not altering the release of activin A, does alter the subsequent release of pro-inflammatory cytokines into the circulation together with the release of endogenous follistatin suggesting, firstly, that activin is important in regulating cytokine release in acute inflammatory responses and, secondly, that activin stimulates the release of follistatin as part of a short loop feedback system.

Activin is known to be involved in the inflammatory process, but its precise role is not clear. We have documented that activin is released rapidly into the circulation in response to, for instance, a signal produced by the bacterial product lipopolysaccharide (LPS). If follistatin is administered around the time when inflammation is induced, then activin's activities are automatically reduced and certain key signals important to inflammation are altered. These include the inflammatory "cytokines", tumour necrosis factor α, and interleukin 6. The present inventors have determined that administration of activin and/or follistatin will modify production of these key cytokines. This process could be harnessed in patients to beneficially modify the inflammatory response in key diseases such as septicemia to assist in the treatment process.

The present inventors provide here the first set of data to show that activin can modulate the release of cytokines *in vivo* (in other words, in an intact organism). The data shows that activin can alter the levels of key pro-inflammatory cytokines released into the circulation in response to inflammation stimulated by the bacterial product LPS.

In a first aspect the present invention provides a method of treating an inflammatory condition comprising administering an effective amount of activin A and/or follistatin to a patient in need of such treatment.

In a preferred embodiment the inflammatory condition is an LPS-induced inflammatory condition.

The association of activin A release with LPS specifically highlights the applicability of activin A and follistatin as therapeutics in the treatment of inflammatory and potentially infectious diseases such as sepsis and meningitis.

Experimental evidence indicates that activin A release occurs directly in response to inflammatory challenge with LPS. Follistatin, the activin binding protein, is released later than activin A in response to LPS indicating that the activin A released is not bound by follistatin and therefore has not been neutralized allowing it to mediate its effects. Further, activin A is still released when follistatin is administered before LPS while follistatin release is suppressed by the administration of follistatin prior to LPS, indicating that activin stimulates the release of follistatin as part of a short-loop feedback system.

Furthermore, the level of the pro-inflammatory cytokine tumour necrosis factor-α (TNFα) released into the circulation is significantly suppressed when follistatin is administered prior to LPS compared to administration of LPS alone. Thus activin modulates the release of cytokines that are an essential part of a normal inflammatory response. TNFα has also been highlighted as a potential harmful cytokine when released in too great an amount or in the wrong tissue. Therefore, follistatin is indicated as useful in dampening an aberrant inflammatory response by reducing TNFα release.

Administration of follistatin prior to LPS also alters both the temporal release and the amount of Interleukin-6 compared to LPS alone, identifying a role for activin A in the treatment of a subtle inflammatory response, for example, the need to fight off an infection as occurs in sepsis.

Finally the release of IL-1β is also altered by prior administration of follistatin before LPS compared to LPS alone, further highlighting the ability of activin to modulate the release of key pro-inflammatory cytokines.

In a second aspect the present invention provides a method of dampening an aberrant inflammatory response in a patient in need of such treatment which method comprises administering an effective amount of follistatin to the patient.

In a third aspect the present invention provides a method of treatment of a subtle inflammatory response in a patient in need of such treatment which method comprises administering an effective amount of activin to the patient.

In a fourth aspect the present invention provides a method of modulating release of at least one pro-inflammatory cytokine in a patient in need of such treatment which method comprises an effective amount of activin administered to the patient.

In a fifth aspect the present invention provides use of activin and/or follistatin in a method of treating an inflammatory condition comprising administering an effective amount of activin A and/or follistatin to a patient in need of such treatment.

In a preferred embodiment the inflammatory condition is an LPS-induced inflammatory condition.

In a sixth aspect the present invention provides use of follistatin in a method of dampening an aberrant inflammatory response in a patient in need of such treatment which method comprises administering an effective amount of follistatin to the patient.

In a seventh aspect the present invention provides use of activin in a method of treatment of a subtle inflammatory response in a patient in need of such treatment which method comprises administering an effective amount of activin to the patient.

In an eighth aspect the present invention provides use of activin in a method of modulating release of at least one pro-inflammatory cytokine in a patient in need of such treatment which method comprises an effective amount of activin administered to the patient.

In a ninth aspect the present invention provides use of activin and/or follistatin in the manufacture of a medicament for use in a method of treating an inflammatory condition comprising administering an effective amount of activin A and/or follistatin to a patient in need of such treatment.

In a preferred embodiment the inflammatory condition is an LPS-induced inflammatory condition.

In a tenth aspect the present invention provides use of follistatin in the manufacture of a medicament for use in a method of dampening an aberrant inflammatory response in a patient in need of such treatment which method comprises administering an effective amount of follistatin to the patient.

In an eleventh aspect the present invention provides use of activin in the manufacture of a medicament for use in a method of treatment of a subtle inflammatory response in a patient in need of such treatment which method comprises administering an effective amount of activin to the patient.

In a twelfth aspect the present invention provides use of activin in the manufacture of a medicament for use in a method of modulating release of at least one pro-inflammatory cytokine in a patient in need of such treatment which method comprises an effective amount of activin to the patient.

In a thirteenth aspect the present invention provides activin and/or follistatin or a pharmaceutical composition comprising an effective amount of activin and/or follistatin together with at least one pharmaceutically acceptable carrier, excipient or diluent when used in treating an inflammatory condition in a patient in need of such treatment.

In a preferred embodiment the inflammatory condition is an LPS-induced inflammatory condition.

In an fourteenth aspect the present invention provides follistatin or a pharmaceutical composition comprising an effective amount of follistatin together with at least one pharmaceutically acceptable carrier, excipient or diluent when used in dampening an aberrant inflammatory response in a patient in need of such treatment.

In a fifteenth aspect the present invention provides activin or a pharmaceutical composition comprising an effective amount of activin together with at least one pharmaceutically acceptable carrier, excipient or diluent when used in treatment of a subtle inflammatory response in a patient in need of such treatment.

In a sixteenth aspect the present invention provides activin or a pharmaceutical composition comprising an effective amount of activin together

with at least one pharmaceutically acceptable carrier, excipient or diluent when used in modulating release of at least one pro-inflammatory cytokine in a patient in need of such treatment.

In a seventeenth aspect the present invention provides a method of regulating erythropoiesis, lymphopoiesis and/or the production of acute phase proteins in a patient in need of such treatment which method comprises administering an effective amount of activin to the patient.

In an eighteenth aspect the present invention provides activin or a pharmaceutical composition comprising activin together with at least one pharmaceutically acceptable carrier, excipient or diluent when used in a method of regulating erythropoiesis, lymphopoiesis and/or the production of acute phase proteins in a patient in need of such treatment.

In a nineteenth aspect the present invention provides use of activin in the manufacture of a medicament for use in a method of regulating erythropoiesis, lymphopoiesis and/or the production of acute phase proteins in a patient in need of such treatment.

It will be understood that the formulation of activin and follistatin into medicaments or pharmaceutical compositions is in accordance with standard practice in the pharmaceutical arts as is determination of effective therapeutic doses. Effective doses are determined by routine techniques and take into consideration as is necessary patient parameters such as age, weight, general health and co-morbidities.

Activin and follistatin for use in the methods and formulations of the invention are selected from suitable available forms in accordance with standard practice in the pharmaceutical arts.

It is to be appreciated that alterations and/or additions may be made to the parts and/or embodiments previously described without departing from the spirit or ambit of the invention.

Brief Description of the Accompanying Drawings

Figure 1A shows activin A release following an inflammatory challenge, in the form of lipopolysaccharide (LPS), in mice.

Figure 1B shows follistatin release in response to LPS.

Figure 1C shows TNF-α release in response to LPS.

Figure 1D shows IL-6 release in response to LPS.

Figure 1E shows IL-1β release in response to LPS.

Figure 2A shows activin A release following an injection of LPS in mice that received an injection of recombinant human follistatin-288 (rhfollistatin-288) 30 minutes prior to LPS.

Figure 2B shows release of follistatin in mice following administration of rhfollistatin-288 30 minute prior to LPS.

Figure 2C shows the level of TNFα released in mice following administration of rhfollistatin-288 30 minute prior to LPS.

Figure 2D shows the level of interleukin-6 released following injection of rhfollistatin-288 followed by an injection of LPS.

Figure 2E shows the level of IL-1β released following injection of follistatin followed by LPS.

Best and Other Methods of Carrying Out the Invention

EXAMPLE 1

Materials & Methods

Animals and general experimental details. All experiments were conducted in accordance with the NHMRC Australian Code of Practice for the Care of Animals for Scientific Purposes (1997) and were approved by the Monash University Animal Ethics Committee.

One hundred and twenty six male C57Bl/6 mice (4-8 weeks), were randomly allocated into two groups; Group 1 consisted of nine sub-groups of eight animals (total n = 72) while Group 2 consisted of nine sub-groups of six

animals (n = 54). All animals were kept in standard animal housing with access to food and water throughout the experiment. Lipopolysaccharide (LPS) (*E. coli* serotype 0127:B8, Sigma, St Louis, MO, USA) was purified using a phenol-water extraction method as previously described (18), and administered as an intraperitoneal bolus injection of 100 µg in 100 µl of isotonic, non-pyrogenic saline solution per mouse. Recombinant human follistatin-288 (rhfollistatin-288; Biotech, Australia) was administered as an intraperitoneal injection of 1 µg in 100 µl of isotonic, non-pyrogenic saline solution, 30 minutes prior to LPS. Group 1 received injections of LPS and rhfollistatin-288 while group 2 received an injection of LPS alone. Mice were then anaesthetized with an inhalant form of isoflurane (Abbott Australasia LTD, Kurnell, Australia), and sacrificed for blood collection at 30 minutes, 1, 2, 3, 5, 8, 12 & 24 hours and one group was sacrificed without an injection to act as controls for basal levels. Blood was collected into a 1.5 ml centrifuge tube containing 50µl of ethylenediaminetetra-acetic acid (EDTA, BDH Laboratory Supplies, Poole, UK) and centrifuged at 250 *g* at room temperature with plasma removed and stored at -20°C until assayed for activin A, follistatin, TNFα, IL-6 and IL-1β.

Assays.

Activin A was measured by ELISA as previously described using human recombinant activin A as a standard (19). This ELISA measures both free and follistatin-bound activin and does not cross react significantly with other isoforms of activin (19). The mean sensitivity was 0.01 ng/ml, and the mean intra- and inter-assay coefficients of variation (CVs) were 3.9% and 5.1% respectively.

Follistatin concentrations in serum were measured with a radioimmunoassay as previously described (20). The standard and tracer employed was rhfollistatin-288. As with the Activin A ELISA, this RIA measures both free and bound forms of follistatins. The mean assay sensitivity was 2.7 ng/ml, ED_{50} was 13.3 ng/ml, and the intra- and inter-assay CVs were 6.4% and 10.2%, respectively.

Mouse cytokines TNFα, IL-6 and IL-1β were measured by ELISA (R&D Systems, Minneapolis, MN, USA). These assays use mouse recombinant proteins as standards and monoclonal antibodies for detection. The sensitivity of the TNFα assay was 0.5 ng/ml, and the intra and inter-assay CVs were <10%. The sensitivity of the IL-6 assay was 0.2 ng/ml and the intra and inter-assay CVs were < 10% and 12%, respectively. The sensitivity of the IL-1β was ng/ml and the intra and inter-assay CVs were < 10% and < 11%, respectively.

Data analysis

All data was analysed using a one way ANOVA with a paired t- test used to compare differences between time points in the different treatment groups.

Results

The role of activin A in mice following an intraperitoneal LPS challenge.

A robust release of activin A was observed in the mice following an injection of re-extracted LPS. Levels of activin A increased within 30 minutes following LPS administration and peaked at 1 hour returning to baseline levels between 3 to 8 hours, followed by a subsequent increase at 12 hours before returning again to baseline levels at 24 hours (Fig. 1 A). Following LPS administration, follistatin was released into the circulation but was delayed compared to activin A, increasing at 3 hours and remaining elevated until 24 hours (Fig. 1B). The release of TNFα into the circulation was observed to follow the classic monophasic peak, increasing at 0.5 hours ($p<0.01$) post LPS administration, peaking at 1 hour and returning to basal levels at 5 to 8 hours (Fig. 1C). Serum IL-6 was elevated subsequent to elevations in TNFα, increasing between 1 and 2 hours, peaking at 2 hours ($p<0.01$) and remaining elevated until between 5 ($p<0.01$) and 8 hours (Fig. 1D). The levels of IL-1β in the circulation were significantly lower than TNFα or IL-6 (30-50 fold) with IL-1β increasing 1 hour post injection and peaking at 5 hours ($p<0.01$) before returning to basal levels at 8 hours (Fig. 1E).

The peak release of activin A was unaffected by the administration of rhfollistatin-288. Following LPS administration activin A release into the circulation was still rapid and robust peaking at 1 hour and returning to basal levels within 5 hours (Fig. 2A). Interestingly, the concentration of circulatory mouse follistatin-288 was significantly suppressed over the entire peak period, 5-8 hours ($p<0.03$) following LPS administration in mice injected with rhfollistatin-288 (Fig. 2B). Additionally, TNFα release was significantly suppressed by administration of rhfollistatin-288 prior to injection of LPS ($p<0.01$) although the profile of release was not significantly altered (Fig. 2C). Conversely, IL-6 release was altered in both absolute amounts and temporally. Interestingly, IL-6 peak concentrations were significantly increased ($p<0.01$) in mice administered rhfollistatin-288 prior to LPS by approximately 2 fold (Fig. 2D). Furthermore, increases in IL-6 occurred earlier in the presence of rhfollistatin-288, peaking at 1 hour as compared to 2 hours in mice receiving LPS alone. Release of IL-1β was not as evident in the presence of rhfollistatin-288 when compared to mice that received LPS alone (Fig 2E). Additionally, the profile also shifted such that elevations in serum concentrations occurred earlier in the presence of rhfollistatin-288, peaking at 2 hours compared to 5 hours in mice receiving LPS alone ($p<0.01$). However, it should be noted that there was not a significant difference in the concentrations of IL-1β released at any time point.

Discussion

The data presented here are the first to demonstrate the ability of activin A and follistatin to modulate the inflammatory cascade by altering cytokine release *in vivo*. This has important implications for the role that activin plays in the inflammatory response and in inflammatory disease such as septicemia and meningitis in which activin A and follistatin are found in elevated levels.

It has been previously demonstrated that activin A is released *in vitro* in response to LPS in culture from monocytes, bone marrow stromal cells, macrophages and pregnancy membranes and also in response to inflammatory cytokines including TNFα and IL-1 (7, 21-24). The majority of these reports

characterized this activin A response in relation to haematopoiesis/erythropoiesis and suggest that activin A may be important in host defence by stimulating erythropoiesis. However, no previous *in vivo* data exists which suggest that activin A is capable of modulating inflammatory cascades.

The present data indicates that activin A stimulates the release of TNFα. Previously, activin A was shown to stimulate the release of inflammatory mediators from rat derived bone marrow macrophages including TNFα, IL-1 and prostaglandins (25). In this study, blockade of activin altered the levels of cytokines released; it also altered the temporal expression of both IL-6 and IL-1β, indicating that either activin is involved directly in regulating the release of IL-6 and IL-1β or alternatively, the concentration of TNFα and/or other early cytokines are critical to the release of downstream cytokines in an inflammatory cascade. Currently, limited data exists demonstrating the ability of activin to alter the release of the cytokines IL-6 and IL-1β.

Beyond the limited data highlighting the ability of activin A to regulate the production of certain cytokines, a number of studies have addressed the ability of activin A to modulate the bioactivity of these cytokines. The antagonism of IL-6 by activin A has been studied *in vitro*, including the ability of activin A to antagonize the IL-6-stimulated production of acute phase proteins, a key process in a normal inflammatory response, an effect which could be blocked by the addition of follistatin (11). Activin A is also capable of antagonizing the IL-6-stimulated differentiation and phagocytic activity of M1 myeloblasts and IL-6-induced proliferation of 7TD1 B lymphoid cells (13, 15).

It is apparent that the activin A released in the animal model used here is indeed bioactive as evidenced by the changes in profile induced by blocking its function following rhfollistatin-288 administration. Furthermore, the peak of activin A and follistatin concentrations in serum did not overlap, indicating that activin and follistatin are not released concurrently and that the activin A detected in the assay is not bound to follistatin and is therefore bioactive.

These novel data show activin A together with follistatin are capable of modulating the release of inflammatory cytokines *in vivo*. Furthermore, it supports

a role for activin A and follistatin as therapeutics for the treatment of inflammatory disorders which activin A is already associated with and others where aberrant inflammatory responses take place. Furthermore, these data have implications for a number of processes in the host including erythropoiesis, lymphopoiesis and the production of acute phase proteins, due to activin's ability to regulate these processes. The association of activin A release with LPS specifically highlights the applicability of activin A and follistatin as therapeutics in the treatment of infectious diseases such as sepsis and meningitis.

The animal data provided here is of relevance to the functions of activin and follistatin in humans and therefore provides support for the use of activin and follistatin in the treatments described herein.

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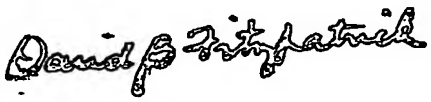
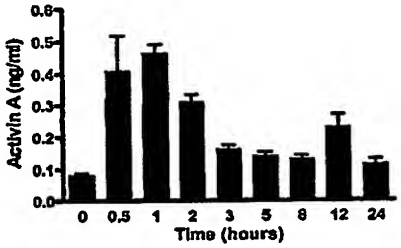


Figure 1A



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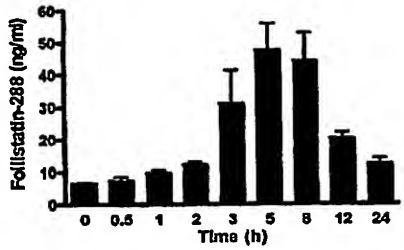

Figure 1C

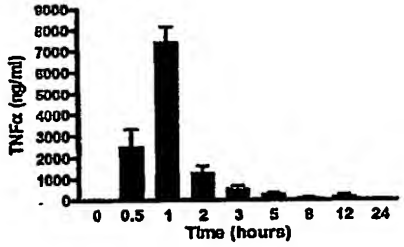

Figure 1D

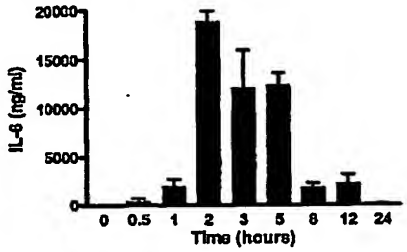

Figure 1E

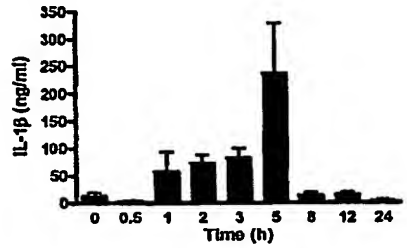

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Figure 2A

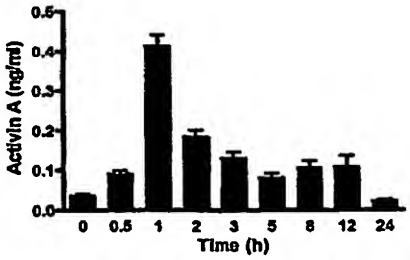


Figure 2B

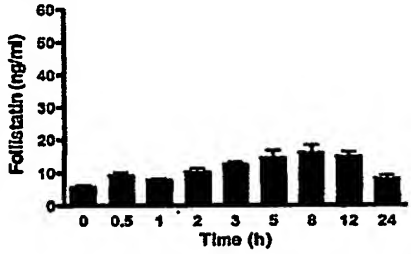


Figure 2C

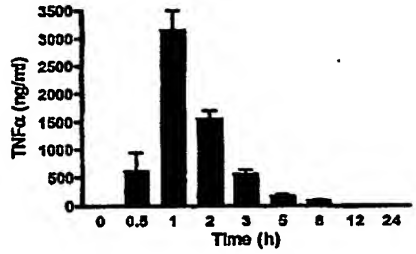


Figure 2D

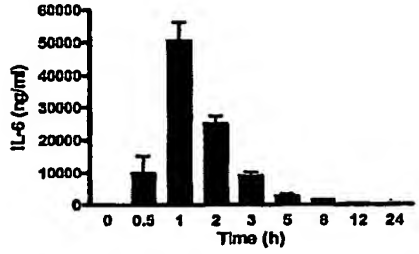
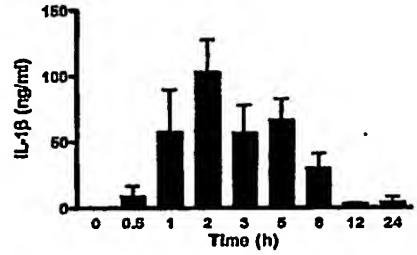


Figure 2E



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